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Iodide Oxidation by a Novel Multicopper Oxidase from the Alphaproteobacterium Strain Q-1

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Alphaproteobacterium strain Q-1 is able to oxidize iodide (I^-) to molecular iodine (I_2) by an oxidase-like enzyme. One of the two isoforms of the iodide-oxidizing enzyme (IOE-II) produced by this strain was excised from a native polyacrylamide gel, eluted, and purified. IOE-II appeared as a single band (51 kDa) and showed significant in-gel iodide-oxidizing activity in sodium dodecyl sulfate-polyacrylamide gel electrophoresis without heat treatment. However, at least two bands with much higher molecular masses (150 and 230 kDa) were observed with heat treatment (95°C, 3 min). IOE-II was inhibited by NaN_3, KCN, EDTA, and a copper chelator, *o*-phenanthroline. In addition to iodide, IOE-II showed significant activities toward phenolic compounds such as syringaldazine, 2,6-dimethoxy phenol, and *p*-phenylenediamine. IOE-II contained copper atoms as prosthetic groups and had UV/VIS absorption peaks at 320 and 590 nm. Comparison of several internal amino acid sequences obtained from trypsin-digested IOE-II with a draft genome sequence of strain Q-1 revealed that the products of two open reading frames (IoxA and IoxC), with predicted molecular masses of 62 and 71 kDa, are involved in iodide oxidation. Furthermore, subsequent tandem mass spectrometric analysis repeatedly detected peptides from IoxA and IoxC with high sequence coverage (32 to 40%). IoxA showed homology with the family of multicopper oxidases and included four copper-binding regions that are highly conserved among various multicopper oxidases. These results suggest that IOE-II is a multicopper oxidase and that it may occur as a multimeric complex in which at least two proteins (IoxA and IoxC) are associated.

Iodine is an essential trace element for humans and animals, and is a constituent of the thyroid hormones thyroxine and tri-iodothyronine. Insufficient iodine in the diet can cause iodine deficiency disorders such as endemic goiter and cretinism (19, 20). Iodine is present in seawater at a concentration of 0.45 μM (50), and the predominant chemical forms are iodide (I^-; oxidation state, −1), iodate (IO_3^-; oxidation state, +5), and organic iodine compounds (14, 35, 44, 50, 53). In the biogeochemical cycling of iodine, microorganisms may play important roles, together with marine algae and phytoplankton, through the production (1, 2) and remineralization (27, 28) of organic iodine compounds and the redox changing of inorganic iodine species, i.e., iodide and iodate (3, 4). Oxidation of iodide is an important process in the redox cycling of iodine, but little is known about the mechanism of this reaction in nature. Wong (50, 51) suggested that autooxidation of iodide to iodate does not occur in seawater, since the first step of iodide oxidation, i.e., oxidation of iodide to molecular iodine (I_2; oxidation state, 0), is thermodynamically unfavorable. However, subsequent oxidation of I_2 to form iodate may occur spontaneously, suggesting that the oxidation of iodide to I_2 is mediated biologically in nature (52). Although marine algae such as *Laminaria* are known to oxidize iodide using a cell wall haloperoxidase (25), there are still many uncertainties surrounding iodide oxidation by other organisms, especially by microorganisms.

Previously, we screened for the presence of iodide-oxidizing microorganisms in different environments and found that certain heterotrophic bacteria isolated from iodide-rich natural gas brine water were able to oxidize iodide to I_2 (4). They were phylogenetically divided into two groups (groups A and B) within *Alphaproteobacteria*. Group A was most closely related to *Roseovarius tolerans*, with 16S rRNA gene sequence similarities of 94 to 98%, while group B was distantly related to *Rhodothalassium salexigens*, with sequence similarities of 89 to 91%. Such iodide-oxidizing bacteria were repeatedly isolated from brine water, which is a unique environment containing a very high level of iodide (0.06 to 1.2 mM), but soil and seawater samples did not yield any isolates. Interestingly, iodide-oxidizing bacteria were successfully isolated from natural seawater samples, if the samples had been incubated with 1 mM iodide for a week to several months. After incubation, the population size of iodide-oxidizing bacteria in the samples increased to 10^3 to 10^5 CFU ml^{-1}, whereas, before incubation, the population size was below the detection limit(<10 CFU ml^{-1}) (4).

Iodide-oxidizing activity was detected in the culture supernatant of iodide-oxidizing bacteria (4). The activity was not dependent on H_2O_2 but was completely inhibited under anaerobic conditions. These results suggest that an extracellular oxidase is involved in iodide oxidation. In the present study, we purified the iodide-oxidizing enzyme (IOE) from a group B iodide-oxidizing bacterial strain, Q-1, and characterized its enzymatic properties. In addition, the draft genome sequence of strain Q-1 was determined in an attempt to identify putative IOE genes encoding peptide sequences that were recovered from trypsin-digested IOE.

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MATERIALS AND METHODS

Bacterial strain and culture conditions. Alphaproteobacterium strain Q-1, previously isolated from iodide-rich natural gas brine water in Miyazaki prefecture, Japan (4), was used in the present study. The strain was grown aerobically in marine broth 2216 (Becton Dickinson, Sparks, MD) at 30°C. In most cases, the medium was supplemented with 40 μM $CuCl_2 \cdot 2H_2O$.

Enzyme assays. In the presence of high concentration of iodide, I_2 forms tri-iodide (I_3^-), which has a high extinction coefficient of 25.5 mM^{-1} cm^{-1} at 353 nm. Here, the IOE activity was determined by measuring I_3^- formation (4). The reaction mixture contained the enzyme preparation (culture supernatant or purified enzyme), 20 mM sodium acetate buffer (pH 5.5), and 10 mM KI. The reaction was started by the addition of the enzyme preparation at 30°C. After 20 min of incubation, I_3^- formation was measured spectrophotometrically. One unit of IOE was defined as the amount of enzyme required to form 1 μmol of I_3^- per min.

All assays to determine the oxidation of various substrates were performed at 30°C in 20 mM sodium acetate buffer (pH 5.5). One unit was defined as the amount of enzyme that catalyzed the formation of 1 μmol of the appropriate product per min in all cases. The molar absorption coefficients used for these substrates were as follows: syringaldazine [*N*,*N*′-bis(3,5-dimethoxy-4-hydroxybenzylidene hydrazine)], ε_{525} = 65.0 mM^{-1} cm^{-1}; ABTS [2,2′azinobis(3-ethylbenzthiazolinesulfonic acid)], ε_{420} = 36.0 mM^{-1} cm^{-1}; *p*-phenylenediamine (*p*PD), ε_{487} = 14.7 mM^{-1} cm^{-1}; hydroquinone, ε_{247} = 21.0 mM^{-1} cm^{-1}; *o*-dianisidine, ε_{460} = 27.5 mM^{-1} cm^{-1}; catechol, ε_{392} = 1.46 mM^{-1} cm^{-1}; 2,6-dimethoxy phenol (2,6-DMP), ε_{468} = 27.5 mM^{-1} cm^{-1}; gallic acid, ε_{266} = 7.25 mM^{-1} cm^{-1}; pyrogallol, ε_{450} = 4.40 mM^{-1} cm^{-1}; and guaiacol, ε_{465} = 12.1 mM^{-1} cm^{-1}.

The activity of tyrosinase, which catalyzes both the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-dopa) and the oxidation of L-dopa to dopaquinone, was determined on the basis of auto-oxidation of dopaquinone to the orange pigment dopachrome (ε_{475} = 3.7 mM^{-1} cm^{-1}). The reaction mixture contained 20 mM sodium acetate buffer (pH 5.5) and either 1 mM L-tyrosine or 1 mM L-dopa. Mn(II)-oxidizing activity was qualitatively assayed by observing the formation of brown Mn oxides with 20 mM $MnCl_2$ or $MnSO_4$ as a substrate (13). Peroxidase and haloperoxidase activities were assayed as described previously (4).

Purification of IOE. Strain Q-1 was cultured in marine broth 2216 containing 40 μM Cu^{2+} at 30°C. Culture broth after 48 h was centrifuged at 6,000 × *g* for 20 min at 4°C, and the supernatant was concentrated and desalted by ultrafiltration with an Amicon Ultra centrifugal filter (50K; Millipore, Bedford, MA). The concentrated supernatant was applied to a 10% polyacrylamide gel for electrophoresis under nondissociating conditions (native PAGE). After electrophoresis the gel was incubated in 20 mM sodium acetate buffer (pH 5.5) containing 100 mM KI and 1% soluble starch to visualize the IOE proteins. After this, the bands corresponding to IOE-I and -II were excised, and each band was eluted with 50 mM Tris-HCl buffer (pH 8.0) for 20 h at 4°C. Protein concentrations were determined by BCA protein assay (Thermo Scientific, Rockford, IL) with bovine serum albumin as the standard.

Electrophoresis. The purity of the enzyme was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After the samples were boiled for denaturation with 2% SDS and 5% 2-mercaptoethanol for 3 min, electrophoresis was performed using 7% polyacrylamide gel in 25 mM Tris-glycine buffer (pH 8.3) containing 0.1% SDS by the method described by Laemmli (26). In some cases, electrophoresis was performed without boiling, but with SDS and 2-mercaptoethanol. Precision Plus Protein Dual Color standards (Bio-Rad, Hercules, CA) were used as standard marker proteins. Proteins were visualized by staining with Coomassie brilliant blue (CBB) R-250. Isoelectric focusing (IEF) was performed using gels with pH gradients from 3 to pH 10 (80 by 80 mm, 1-mm thickness, IEF-PAGE Mini; Tefco, Tokyo, Japan) and calibration marker proteins from the Broad pI kit (GE Healthcare, Buckinghamshire, United Kingdom).

Molecular weight estimation. The apparent molecular weight of the native enzyme was estimated by high-performance liquid chromatography (HPLC) through a TSK G3000SW (7.5 mm by 60 cm; Tosoh, Tokyo, Japan) column equilibrated with 20 mM Tris-HCl buffer (pH 7.0) containing 0.3% NaCl. Thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa) were used as the standard marker proteins. The molecular weight of the denatured enzyme was estimated by SDS-PAGE as described above.

UV-visible absorbance spectra. Absorption spectra were determined between 250 and 650 nm at room temperature in 100 mM Tris-HCl buffer (pH 8.0) by using a BioSpec-nano spectrophotometer (Shimadzu, Kyoto, Japan). The purified enzyme was adjusted to 1.5 mg of protein ml^{-1}.

Kinetic constants. The kinetic constants (V_{max}, K_m, and k_{cat}) of the purified enzyme were determined for iodide and ABTS under the assay conditions described above. Lineweaver-Burk plots were constructed from the initial rates obtained at various substrate concentrations. The k_{cat} was obtained with the following equation: $k_{cat} = V_{max}/[E]$, where [E] is the enzyme concentration.

Cofactor analysis. For determination of cofactor composition, the purified enzyme was hydrolyzed with sulfuric acid, and metal detection was performed by inductively coupled plasma mass spectroscopy (ICP-MS; 7500 series; Agilent, Santa Clara, CA).

Internal amino acid sequence. Internal amino acid sequence analysis was performed as described by Rosenfeld et al. (38). Briefly, the active IOE-II band was excised from a native PAGE gel and digested with trypsin for 20 h at 35°C, and the resulting peptide fragments were separated using reversed-phase HPLC. The fragments were analyzed by a protein sequencer (Procise 494 HT protein sequencing system; Applied Biosystems, Carlsbad, CA).

Preparation of the DNA library for sequencing. Genomic DNA of strain Q-1 was extracted and purified by DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. DNA was uniformly sheared to 200 bp by using adaptive focused acoustics (Covaris, Woburn, MA). We constructed a DNA library with a median insert size of 200 bp for a paired-end read format. The quality of the DNA library was checked with the Sanger method by *Escherichia coli* transformation of aliquots of the library solution. The library was sequenced on a genome analyzer II (Illumina, San Diego, CA). Sample preparation, cluster generation, and paired-end sequencing were carried out according to the manufacturer's protocols with minor modifications (Illumina paired-end cluster generation kit GAII v2, 36-cycle sequencing kit, v3). Image analysis and ELAND alignment were performed with Illumina Pipeline Analysis software (v1.6). Sequences passing standard Illumina GA Pipeline filters were retained. We obtained 584 Mb of total read bases with 11,698,620 read sequences.

***De novo* assembly of the read data and annotation of the coding regions in contigs.** Read sequences were assembled *de novo* with the Velvet assembly program (55). For optimization of the hash value of the assembly process, we used the N50 size. Finally, 306 contigs were assembled with 3.09 Mb of total bases in the contigs. Assembled contigs were annotated by the bacterial annotation pipeline b-MiGAP (46). Each CDS (CoDing Sequence) was annotated by BLAST search of the databases RefSeq, TrEMBL, and NR. Finally, 2,554 CDSs longer than 100 amino acid residues were called by b-MiGAP from the contigs.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Protein sequencing using MS was carried out as described by Shevchenko et al. (45). The tryptic digest of IOE-II, prepared as described above, was directly analyzed by nanoscale HPLC (Paradigm MS2; Michrom Bioresources, Auburn, CA) with an L-column ODS (0.1 by 50 mm; Chemical Evaluation and Research Institute, Tokyo, Japan). LC was coupled to a tandem mass (MS/MS) spectrometer (Q-Tof2; Waters Mi-

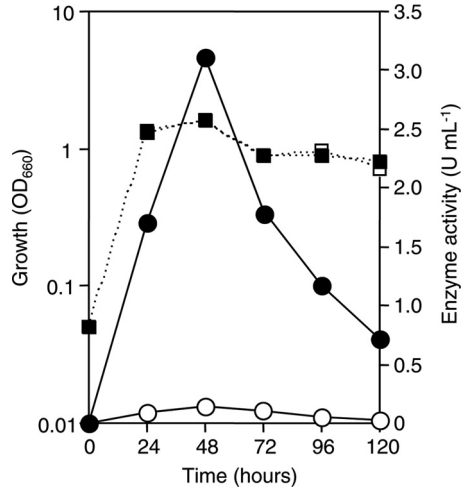

FIG 1 Enhanced production of IOE by Cu^{2+} ion. Strain Q-1 was cultured aerobically in marine broth 2216 with (closed symbols) or without (open symbols) 40 μM Cu^{2+} ion. Cell growth (squares) and IOE activity in the culture supernatant (circles) are shown.

cromass, Manchester, United Kingdom) equipped with a nano-electrospray ionization source. Positive ion tandem mass spectra were measured.

Phylogenetic analysis. The *ioxA* gene sequence was translated to the amino acid sequence, which was aligned with reference sequences by using the CLUSTAL X program, version 2.1. The phylogenetic tree was constructed by the neighbor-joining method (40), and bootstrap values were obtained from data resampling of 1,000 replicates.

Nucleotide sequence accession numbers. The sequence of the gene cluster surrounding the *ioxA* and *ioxF* genes was deposited in GenBank under accession number AB693882.

RESULTS

Enhanced production of IOE with Cu^{2+} ion. When strain Q-1 was cultured in marine broth 2216, maximum production of IOE in the supernatant (0.15 U·ml^{-1}) was observed at 48 h (Fig. 1). IOE production was not induced by iodide, and the addition of 1 mM iodide inhibited both growth and enzyme production, probably because of the toxicity of I_2 (data not shown). Thus, strain Q-1 was cultured without iodide in the following experiments. When the culture medium was supplemented with 40 μM Cu^{2+} ion, enzyme production was 20 times higher (3.1 U·ml^{-1}) than in the medium without Cu^{2+} ion (Fig. 1). More than 80 μM Cu^{2+} ion inhibited enzyme production. No other metal ions (40 μM Fe^{2+}, Ca^{2+}, Mg^{2+}, and Zn^{2+} each) enhanced IOE production.

Purification of IOE-II. Native PAGE analysis of concentrated culture supernatant revealed that the readily visible proteins secreted by strain Q-1 were IOE, and that these IOEs consisted of two isoforms, designated IOE-I and IOE-II (Fig. 2A). Occasionally, one more band was observed near IOE-I, but it did not appear to have IOE activity (Fig. 2A). Attempts to purify each isoform by $(NH_4)_2SO_4$ precipitation, followed by several chromatographic separations (anion exchange, gel filtration, and hydrophobic interaction chromatography), were unsuccessful, since a significant increase in specific activity was not observed, and the two isoforms were not separated. Therefore, we decided to isolate each isoform from a native PAGE gel by cutting out the active regions. Culture supernatant containing IOE with a specific activity of 2.85 U mg^{-1} was concentrated three times by ultrafiltration (with cutoff size of 50 kDa) and applied to native PAGE. After activity staining with iodide and starch, the purple bands corresponding to IOE-I and -II were excised, and each was eluted. Although IOE-I did not show significant activity after elution, 0.284 mg of IOE-II with a

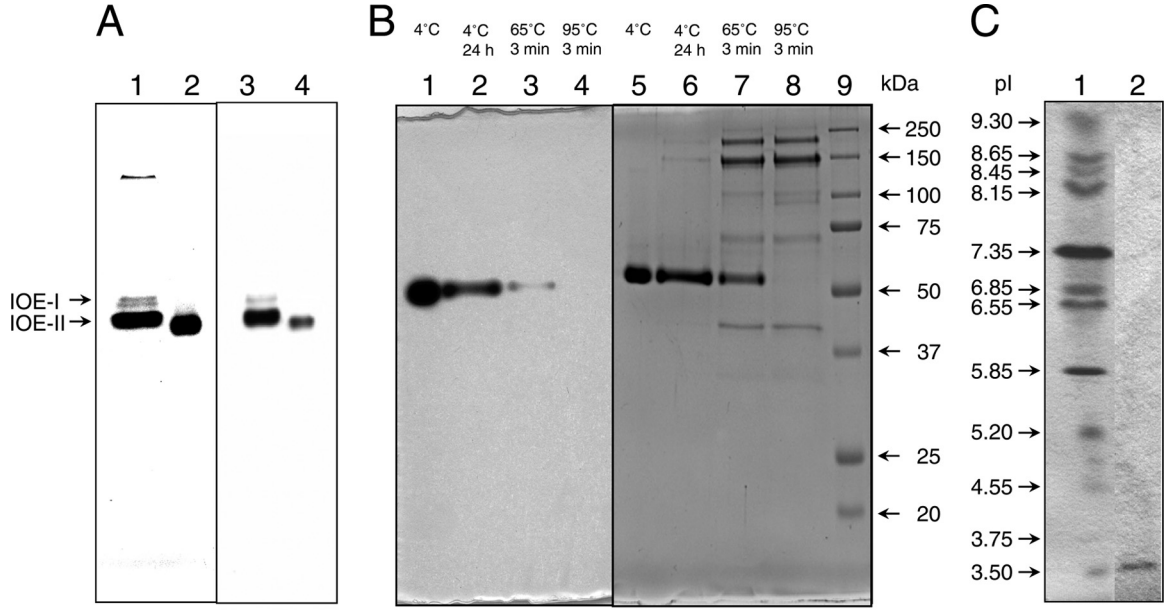

FIG 2 Native PAGE (A), SDS-PAGE (B), and IEF-PAGE (C) analyses of IOE-II. (A) Concentrated culture supernatant (lanes 1 and 3) and purified IOE-II (lanes 2 and 4) were run on native PAGE gels, and the gels were stained with CBB (left) for total proteins or with iodide and starch (right) for activity assay. (B) Purified IOE-II was run on SDS-PAGE gels with or without heat treatment, and the gels were stained with iodide and starch (left) or with CBB (right). Lanes 1 and 5, IOE-II was mixed with SDS and 2-mercaptoethanol, and the mixture was immediately applied on the gels without heat treatment; lanes 2 and 6, the mixture was stored at 4°C for 24 h and was applied without heat treatment; lanes 3 and 7, the mixture was heated at 65°C for 3 min before application; lanes 4 and 8, the mixture was heated at 95°C for 3 min before application; lane 9, size standard. (C) IOE-II was run on an IEF-PAGE gel (lane 2). Lane 1 represents the standard marker proteins.

TABLE 1 Summary of purification of IOE-II from strain Q-1[a]

Purification procedure	Protein (mg)	Total enzyme activity (U)	Sp act (U mg^{-1})	Yield (%)	Purification (fold)
Culture supernatant	9.41	26.8	2.85	100	1.00
Concentration by ultrafiltration	2.84	24.0	8.45	89.6	2.97
Elution of IOE-II from excised active band	0.284	6.26	22.1	23.4	7.75

[a] Representative data from five independent experiments are shown.

specific activity of 22.1 U mg^{-1} was successfully obtained (Table 1). From 12.0 ml of culture supernatant containing 9.41 mg of protein, IOE-II was purified 7.75-fold with a final yield of 23.4%. Purified IOE-II showed a slightly different mobility from crude IOE-II on native PAGE gel (Fig. 2A).

When purified IOE-II was analyzed by SDS-PAGE without heat treatment (but using 2-mercaptoethanol), a single band with a molecular mass of 51 kDa was observed (Fig. 2B). This band still showed in-gel iodide-oxidizing activity, indicating that IOE-II is resistant to SDS and reducing reagent. On an SDS-PAGE gel with heat treatment (95°C, 3 min), however, two bands with much higher molecular masses of approximately 150 and 230 kDa were observed (Fig. 2B). Under this condition, faint bands of 39, 68, and 86 kDa were also observed. The profile of the SDS-PAGE gel did not change if the denaturation time was extended to 30 min or if the sample solution was boiled with excess urea (data not shown). When the denaturation temperature was decreased to 65°C, three bands with molecular masses of 51, 150, and 230 kDa were observed (Fig. 2B). This indicated that the two bands of 150 and 230 kDa were denatured products of the 51-kDa band. IOE-II also appeared as a single band on IEF-PAGE gels, and its isoelectric point was 3.6 (Fig. 2C). The apparent molecular mass of native IOE-II, determined by gel filtration chromatography, was 155 kDa with a single peak. These results suggest that IOE-II may be a high-molecular-mass protein. Since only a single band or peak was observed in native PAGE, SDS-PAGE (without heat treatment), IEF-PAGE, and gel filtration chromatography, we concluded that IOE-II was homogeneously purified.

Biochemical characterization of IOE-II. ICP-MS analysis revealed that IOE-II contained copper and zinc atoms. Based on the estimated molecular mass of 155 kDa in gel filtration chromatography, the presence of 3.96 copper atoms and 4.90 zinc atoms per protein molecule was estimated. The UV-visible spectrum of IOE-II displayed a significant peak at 590 nm, and a shoulder at 320 nm (Fig. 3). These peaks inferred the presence of types 1 and 3 copper centers, respectively (see below in detail).

The activity of IOE-II was significantly inhibited by sodium azide (90 and 98% inhibition at 0.1 and 1 mM, respectively), EDTA (48 and 99% inhibition at 0.1 and 1 mM, respectively), potassium cyanide (15 and 66% inhibition at 1 and 10 mM, respectively), and the copper chelator *o*-phenanthroline (17 and 61% inhibition at 1 and 10 mM, respectively). The activity was not affected by 1 mM (each) Ca^{2+}, Mg^{2+}, Zn^{2+}, and Mn^{2+} ions, but 1 mM Fe^{2+} and Cu^{2+} ions showed 47 and 17% inhibition, respectively.

The effect of pH on the activity and stability of purified IOE-II was examined with 20 mM citrate-NaOH buffer (pH 3.0 to 5.0), 20 mM acetate-NaOH buffer (pH 4.0 to 5.5), 20 mM potassium phosphate buffer (pH 6.0 to 8.0), 20 mM Tris-HCl buffer (pH 7.0 to 9.0), and 20 mM glycine-NaOH buffer (pH 9.0 to 10.0) (see Fig. S1A in the supplemental material). The optimum pH was 5.5 in acetate-NaOH buffer. The activity was relatively stable under alkaline conditions after 30 min of incubation at 4°C and was most stable at pH 6.0 in phosphate buffer (see Fig. S1B in the supplemental material). The temperature stability was measured after 30-min treatment at various temperatures. The activity of IOE-II gradually decreased at temperatures higher than 40°C, while the optimum temperature was 30°C (see Fig. S1C in the supplemental material).

IOE-II had significant activities toward ABTS and *p*PD (Table 2), both of which are common substrates for laccases. The activity with *p*PD was much higher than that with ABTS. Activity stain with ABTS and *p*PD on native PAGE gels verified that IOE-II possessed these activities (data not shown). Laccases are multicopper oxidases that catalyze the oxidation of phenolic compounds using molecular oxygen (33, 41). Many phenolic compounds were then examined as potential substrates for IOE-II (Table 2). The enzyme showed significant activities for syringaldazine, 2,6-DMP, hydroquinone, and *o*-dianisidine. IOE-II was not able to oxidize manganese ion and showed neither peroxidase nor haloperoxidase activity.

When iodide was used as the substrate, IOE-II showed K_m and k_{cat} values of 2.64 mM and 2.48×10^3 min^{-1}, respectively (Table 3). On the other hand, K_m and k_{cat} values of 3.85 μM and 8.96×10^2 min^{-1}, respectively, were observed when ABTS was the substrate.

Internal amino acid sequences of IOE-II. We excised two bands with high molecular masses (150 and 230 kDa) from an

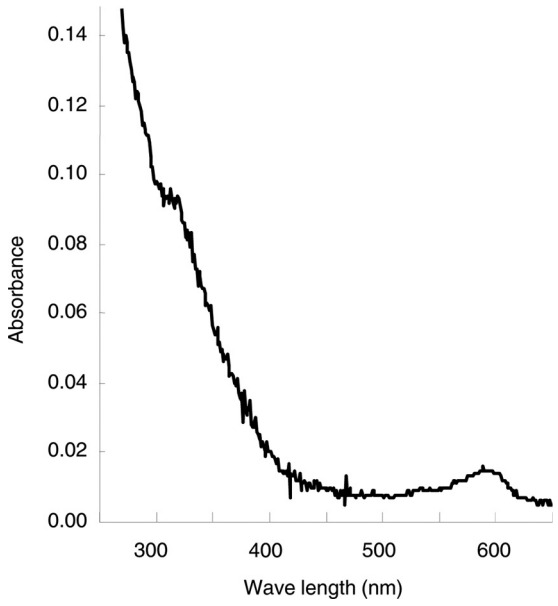

FIG 3 UV-visible absorbance spectrum of IOE-II.

TABLE 2 Substrate specificity of the purified IOE-II

Substrate	Substrate concn (mM)	Mean relative activity (%) ± SD[a]
Iodide	10	100
ABTS	0.1	9.0 ± 0.2
*p*PD	15	69.5 ± 5.2
Syringaldazine	0.075	3.2 ± 0.5
2,6-DMP	0.15	0.6 ± 0.0
Hydroquinone	0.2	8.2 ± 0.2
o-Dianisidine	0.15	8.4 ± 1.1
Catechol	1	0
Galic acid	0.1	0
Pyrogallol	1	0
Guaiacol	1	0
$K_4[Fe(CN)_6]$	0.2	0
L-Tyrosine	1	0
L-Dopa	1	0

[a] Means from triplicate determinations are shown.

SDS-PAGE gel with heat treatment and determined their N-terminal amino acid sequences. However, repeated trials were unsuccessful, probably because both of these bands had blocked N termini or included several proteins. Therefore, we digested purified IOE-II with trypsin and analyzed its internal amino acid sequences. Three distinct peptides (MPVAQSVXQ, KDVVELGPG DEV, and LAAIGMIGDL) were successfully recovered, although X represents an unidentified residue. A search of the BLASTP database indicated that the second and third peptides shared homologies with a putative multicopper oxidase (NCBI accession number ZP_01035800) and hypothetical protein ROS217_06439 (ZP_01035798) of *Roseovarius* sp. strain 217, respectively.

Draft genome sequence analysis and identification of putative genes for IOE-II. To further understand the molecular aspects of IOE-II, the draft genome sequence of strain Q-1 was determined with an Illumina genome analyzer II. *De novo* assembly of 584 Mb of the total read data by the assemble program Velvet (55) resulted in 306 contigs (see Materials and Methods). The results suggested that the strain Q-1 genome is ~3.09 Mb with a G+C content of 56.1%. Using the bacterial annotation pipeline MiGAP (46), we identified 2,554 putative CDSs in the draft genome.

Three peptide sequences obtained as described above were used to query a BLAST database of the draft Q-1 genome. The first and second peptide sequences (MPVAQSVXQ and KDVVELGP GDEV) were found within the gene product of an open reading frame (ORF) encoding a putative multicopper oxidase. This ORF (1,608 bp, 536 amino acids) was designated *ioxA* (Fig. 4). The predicted molecular mass of the deduced protein (IoxA) was 62 kDa. A BLASTP search of the GenBank database showed that IoxA shared homologies with putative multicopper oxidases or bilirubin oxidases found in the genome-sequencing projects of *Roseovarius* sp. strain 217 (ZP_01035800, 60% identity), *Methylomonas methanica* MC09 (YP_004515128, 59% identity), and *Stackebrandtia nassauensis* DSM44728 (YP_003515051, 44% identity). From TatP analysis (http://www.cbs.dtu.dk/services/TatP-1.0/), IoxA seemed not to contain an N-terminal signal sequence.

Multicopper oxidases are characterized by three different types of copper-binding sites, i.e., type 1, 2, and 3 copper centers (33, 41). These redox centers enable the protein to couple four one-electron oxidation reactions to the four-electron reduction of oxygen to water. As shown in Fig. 5, each of the cysteine and histidine residues predicted to bind copper to each of the copper centers (types 1 to 3) were present in IoxA. Phylogenetic analysis demonstrated that IoxA is distantly related to previously known bacterial multicopper oxidases such as CotA, CueO, CumA, and CopA (Fig. 6). It formed a clade with putative multicopper oxidases and bilirubin oxidases found in the genome-sequencing projects of various bacteria.

Analysis of the flanking region of *ioxA* revealed that six possible ORFs (*ioxA*, *ioxB*, *ioxC*, *ioxD*, *ioxE*, and *ioxF*) were present with the same orientation and with a relatively close sequential arrangement (Fig. 4). Whether these genes reside in an operon and are cotranscribed remains unknown. The third peptide sequence recovered from IOE-II (LAAIGMIGDL) was found within a deduced protein encoded by *ioxC* (1,932 bp, 644 amino acids), which was located downstream of *ioxA* (Fig. 4). The predicted molecular mass of IoxC was 71 kDa, and it exhibited homologies with the hypothetical proteins of *Roseovarius* sp. strain 217 (ZP_01035798, 42% identity), *M. methanica* MC09 (YP_004515130, 43% identity), and *S. nassauensis* DSM44728 (YP_003515052, 37% identity). TatP analysis predicted that IoxC contained a potential twin-arginine translocational (TAT) signal peptide.

The proteins encoded by *ioxB*, *ioxD*, and *ioxF* all showed homologies with SCO1/SenC family proteins, which are known to bind copper and be involved in the assembly of the cytochrome *c* oxidase complex in yeast (43). In IoxD and IoxF, the conserved copper-binding domains (CXXXC) were also found. The deduced protein encoded by *ioxE* showed homology with a hypothetical protein of *Roseovarius* sp. strain 217 (ZP_01035796, 38% identity). It is noteworthy that *iox* gene cluster arrangement found in strain Q-1 (*ioxA*, *ioxB*, *ioxC*, *ioxD*, and *ioxE*) is very similar to that surrounding the IoxA homolog in *Roseovarius* sp. strain 217 (ZP_01035800, ZP_01035799, ZP_01035798, ZP_01035797, and ZP_01035796).

TABLE 3 Apparent kinetic constants of several multicopper oxidases for iodide and ABTS[a]

Enzyme source	Substrate	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} M^{-1})	Source or reference
IOE-II (strain Q-1)	Iodide	2.64	2.48×10^3	9.41×10^5	This study
	ABTS	3.85×10^{-3}	8.96×10^2	2.32×10^8	
Laccase (*Trametes multicolor*)	Iodide	4.83×10^2	6.42×10^2	1.32×10^3	29
	ABTS	1.41×10^{-2}	3.06×10^4	2.17×10^9	
Laccase (*Myceliophthora thermophila*)	Iodide	1.60×10^2	2.70	1.69×10	54
	ABTS	5.60×10^{-3}	1.46×10^3	2.61×10^8	

[a] Means from three independent experiments are shown.

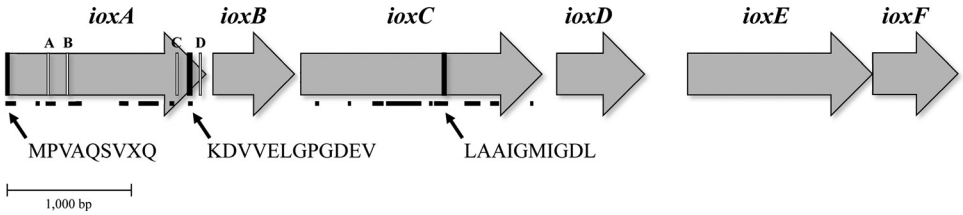

FIG 4 Schematic representation of *iox* genes of the strain Q-1. The locations of putative copper-binding regions are indicated by white bars and labeled A to D according to the data in Fig. 5. The locations of the three peptides detected by internal amino acid sequence analysis are indicated by black bars with arrows and corresponding sequences. The locations of peptides detected by LC-MS/MS analysis of IOE-II are shown with black dots below the *iox* genes.

LC-MS/MS analysis of IOE-II. The peptides from the digestion of the IOE-II band with trypsin were separated by nanoscale reverse-phase chromatography and directly analyzed by nanoelectrospray ionization-tandem mass spectrometry. A database search of tandem mass spectra with a Mascot Search Program revealed that 14 different IoxA peptides and 16 different IoxC peptides were consistently recovered from the active IOE-II band (Fig. 4 and see Table S1 in the supplemental material). The overall coverage of the IoxA and IoxC protein sequences with peptides identified by MS/MS was 32 and 40%, respectively.

DISCUSSION

Heme- or vanadate-containing haloperoxidases oxidize halide ions in the presence of H_2O_2 (30, 47). Heme-containing haloperoxidases include chloroperoxidase (from the fungus *Caldariomyces fumago*), lactoperoxidase, and horseradish peroxidase; vanadate-containing haloperoxidases are represented by vanadate-dependent chloro- and bromoperoxidases found in various fungi and marine algae (49). Although haloperoxidases are actually capable of I_2 production from iodide *in vitro*, the final product *in vivo* may not be I_2, but iodinated organic compounds, since haloperoxidases usually catalyze the halogenation reaction in the presence of appropriate organic substrates such as tyrosine (47). In fact, studies of the biological production of I_2 are very scarce, except for that by marine kelps (24, 25). Previously, we isolated iodide-oxidizing bacteria, which produce I_2 from iodide, from iodide-rich natural gas brine water and iodide-enriched seawater (4). Preliminary characterization of the extracellular iodide-oxidizing activity revealed that the activity was not due to haloperoxidase but due to oxidase. Since there have been few reports on oxidases with iodide-oxidizing activity, we purified one of the isozymes from the group B iodide-oxidizing bacterial strain Q-1 and characterized its biochemical properties.

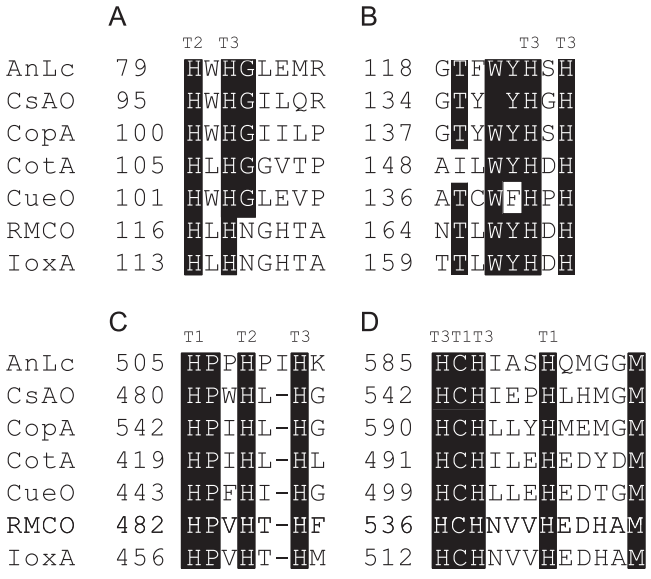

FIG 5 Alignment of the putative copper-binding regions of IoxA and other multicopper oxidases. The amino acid residues corresponding to potential copper-binding sites are indicated as follows: type 1, T1; type 2, T2; and type 3, T3. Highly conserved amino acid residues that are common in more than five multicopper oxidases among the seven aligned enzymes are shaded. Abbreviations: AnLc, *Aspergillus nidulans* laccase (accession no. CAA36787); CsAO, *Cucumis sativus* ascorbate oxidase (CBY84386); CopA, *Pseudomonas syringae* CopA (AAA25806); CotA, *Bacillus subtilis* CotA (BAA22774); CueO, *Escherichia coli* CueO (ZP_03003336); RMCO, *Roseovarius* sp. strain 217 multicopper oxidase (ZP_01035800).

Production of IOE by strain Q-1 was not induced by iodide, but by Cu^{2+} ion. In the presence of 40 μM Cu^{2+} ion, enzyme production was enhanced 20-fold, whereas growth was not affected. ICP-MS analysis revealed that the enzyme contained copper atoms as prosthetic groups. An important role for Cu^{2+} ion was also indicated by the fact that *o*-phenanthroline, a copper chelator, inhibited the activity of IOE-II. Interestingly, IOE-II showed significant activities toward common substrates for laccases, i.e., ABTS, methoxyphenols (syringaldazine and 2,6-DMP), *p*-diphenol (hydroquinone), and aromatic diamines (*p*PD and *o*-dianisidine). Laccases belong to the family of multicopper oxidases, and they have three spectroscopically different copper centers, types 1, 2, and 3 (33, 41). Among these, types 1 and 3 copper centers are characterized by absorption at around 600 and 330 nm, respectively, and the purified IOE-II had absorption peaks around these wavelengths (590 and 320 nm). These results strongly suggest that IOE-II is a multicopper oxidase. Strong inhibition of IOE-II activity by sodium azide also supports the involvement of a multicopper oxidase, since azide bridges types 2 and 3 copper centers and inhibits other bacterial multicopper oxidases, including CueO of *E. coli* (16), laccase of *Azospirillum lipoferum* (11), and various Mn^{2+} oxidases (7, 12, 34, 39). Enhancement of enzyme activity by Cu^{2+} ion has been reported in CueO (16) and YacK (23) of *E. coli* and in the Mn^{2+} oxidases of *Pseudomonas putida* GB-1 (8) and *Bacillus* sp. strain SG-1 (48). Similarly, inhibition by *o*-phenanthroline has been found in Mn^{2+} oxidases of *P. putida* GB-1 (34) and *Erythrobacter* sp. strain SD-21 (12). Our result of cofactor analysis (3.96 copper atoms and 4.90 zinc atoms per protein molecule) also shows a good agreement with the previous reports, in which four copper atoms were found in each multicopper oxidase chain (17, 37). Although it is not common that zinc atoms are included in multicopper oxidases, laccases from *Pleu-*

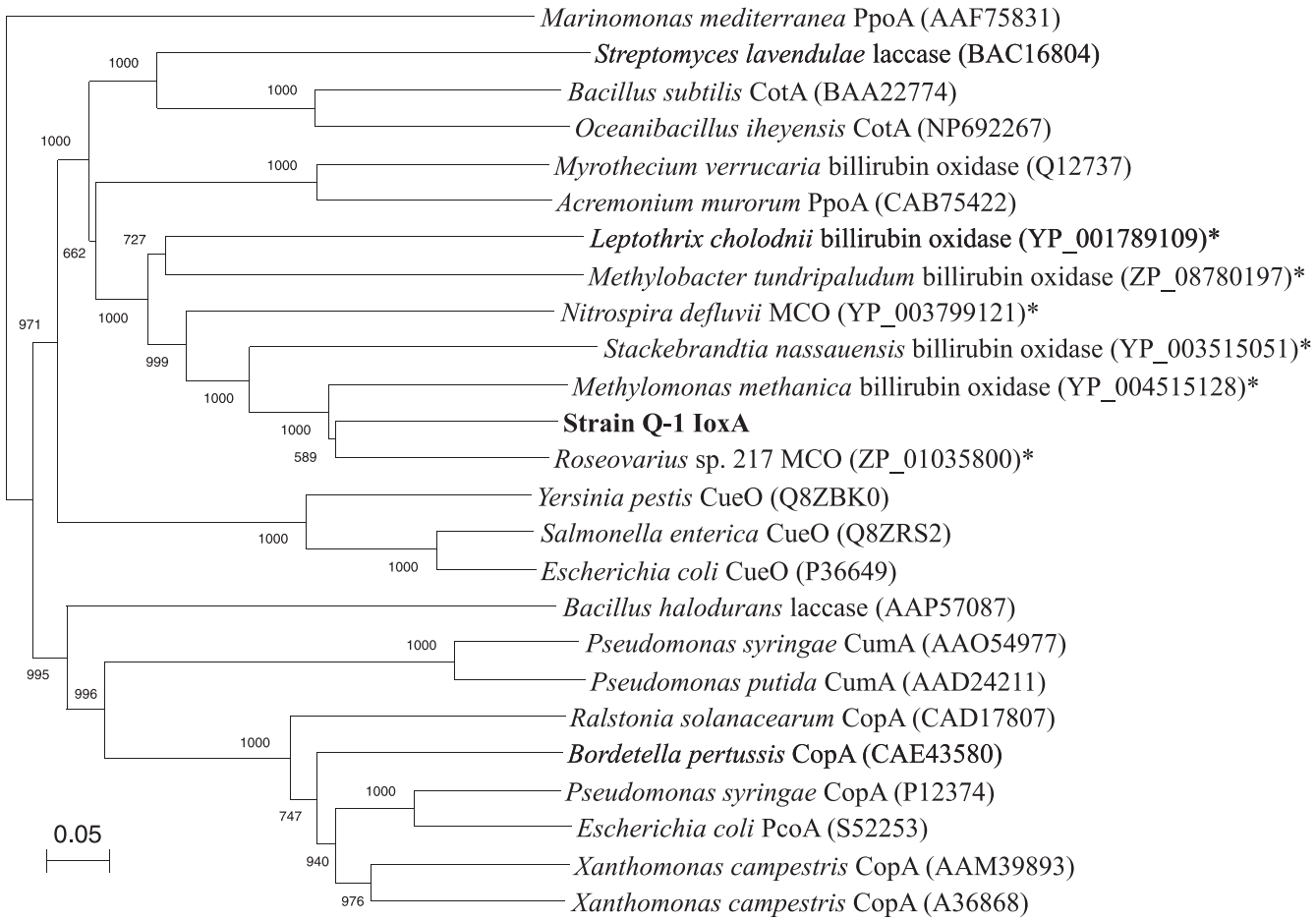

FIG 6 Phylogenetic analysis of translated amino acid sequences of the *ioxA* gene by using the neighbor-joining method. Sequences with an asterisk indicate those found in the genome-sequencing projects of various bacteria without functional analysis. GenBank accession numbers are shown in parentheses. The scale bar represents the number of amino acid changes per site.

rotus ostreatus (36) and *Phellinus ribis* (32) are known to contain two zinc atoms.

To date, there have been only two reports on multicopper oxidases with iodide-oxidizing activity. Xu (54) first found that laccase from the fungus *Myceliophthora thermophila* possessed iodide oxidase activity. Leitner et al. (29) also reported that laccase purified from the white-rot fungus *Trametes multicolor* oxidized iodide as well as a variety of phenolic compounds. However, iodide was a very poor substrate for *T. multicolor* laccase because of a high K_m value (483 mM) and a low k_{cat} value (6.42×10^2 min^{-1}) (29). As shown in Table 3, IOE-II showed a much lower K_m value (2.64 mM) and a much higher k_{cat} value (2.48×10^3 min^{-1}) and thus, the catalytic efficiency (k_{cat}/K_m) for iodide was 3 to 5 orders higher than that for the fungal laccases. Therefore, IOE-II is considered as the most efficient iodide-oxidizing enzyme among the multicopper oxidases reported thus far. Iodide-oxidizing bacteria have been isolated from iodide-rich environments such as natural gas brine water (containing up to 1.2 mM iodide) and seawater enriched with 1 mM iodide (4). Recently, Arakawa et al. (5) found that iodide-oxidizing bacteria became predominant in microcosms comprising natural seawater and 1 mM iodide and suggested that iodide-oxidizing bacteria potentially attack other marine bacteria with I_2 to occupy their ecological niche. Therefore, it is possible that the high catalytic efficiency of IOE contributes to predominant growth of iodide-oxidizing bacteria in iodide-rich environments.

Comparison of several internal amino acid sequences of IOE-II with a draft genome sequence of strain Q-1 revealed that IoxA and IoxC, with predicted molecular masses of 62 and 71 kDa, respectively, were included in IOE-II. In addition, subsequent LC-MS/MS analysis of IOE-II repeatedly detected peptides from these two proteins with high sequence coverage (32 to 40%). These results suggest that at least two proteins, IoxA and IoxC, comprise IOE-II. Sequence analysis demonstrated that IoxA is a typical multicopper oxidase with four copper-binding regions that are highly conserved among various multicopper oxidases. Multicopper oxidases have been found in fungi, plants, and animals, and include laccases, ferroxidases, ascorbate oxidases, and ceruloplasmin (33, 41). There is increasing evidence for the existence of multicopper oxidases in various Gram-positive and Gram-negative bacteria (9, 22). Their possible functions include pigment formation, the utilization of plant phenolic compounds, electron transport, sporulation, copper resistance, and oxidation of metals such as manganese, copper, and iron. To the best of our knowledge, however, no studies have been conducted on bacterial multicopper oxidases capable of iodide oxidation. Furthermore, phy-

logenetic analysis suggests that IoxA is distinct from other known bacterial multicopper oxidases (Fig. 6).

IoxA showed the highest sequence similarity with a multicopper oxidase of *Roseovarius* sp. strain 217, a bacterium originally isolated as a marine methyl halide oxidizer (42). Phylogenetically, *Roseovarius* sp. strain 217 is closely related to known group A iodide-oxidizing bacteria (4) such as strains RB-2A, N213-3, A-6, YS-11, SE-1, and Ka-4, with 16S rRNA gene sequence similarities of 95 to 98% (data not shown). It is thus probable that this strain is also capable of iodide oxidation.

In the present study, a discrepancy between the results of several experiments that measured the molecular mass of IOE-II was apparent. The enzyme appeared as a single 51-kDa band in SDS-PAGE without boiling, whereas at least two bands with much higher molecular masses (150 and 230 kDa) were observed in SDS-PAGE with boiling. In gel filtration chromatography, the apparent molecular mass of IOE-II was estimated to be 155 kDa. Furthermore, the molecular masses of IoxA and IoxC, both of which are proteins detected in IOE-II, were found to be 62 and 71 kDa, respectively. Bacterial multicopper oxidases are often found in high-molecular-mass complexes (9), and several studies have shown differences between the size of the gene and the molecular mass of the enzymatically active product. In the spores of a marine *Bacillus* species, for example, the size of Mn^{2+} oxidase dramatically varied (140 to 205 kDa) among different strains (10). MS/MS analysis of the Mn^{2+} oxidase recovered from an in-gel activity assay repeatedly detected peptides unique to the multicopper oxidase MnxG. However, the length of the *mnxG* gene is nearly identical among these strains, and the predicted molecular mass of MnxG is 138 kDa. Dick et al. (10) suggested that the Mn^{2+} oxidase of *Bacillus* spores may occur as a high-molecular-mass complex because of the association of additional subunits and/or proteins. Since molecular masses greater than 150 kDa were observed both in SDS-PAGE and gel filtration chromatography, IOE-II may also be a high-molecular-mass protein. In addition, repeated detection of peptides from IoxA and IoxC indicates that IOE-II exists as a protein complex in which at least two subunits are associated. However, at present, the accurate molecular mass and the subunit composition of IOE-II remain unclear. Indeed, it is an uncommon feature that 150- and 230-kDa bands are detected in SDS-PAGE under fully denatured conditions (with reducing reagent and boiling), despite the predicted molecular masses of IOE-II subunits being 62 and 71 kDa. Considering the fact that IOE-II still maintained in-gel activity in the presence of SDS and reducing reagent (Fig. 2B), it is possible that IOE-II is highly resistant to denaturation. Therefore, we speculate that the 150- and 230-kDa bands appearing on fully denatured SDS-PAGE gels possibly represent native IOE-II and its aggregated form, respectively.

From an applied point of view, IOE-II could be used as a novel antimicrobial enzyme system. I_2 has antimicrobial activity against a wide variety of microorganisms, including Gram-positive and Gram-negative bacteria, filamentous fungi, yeasts, viruses, and certain bacterial spores (31). Because of its broad spectrum of activity, I_2 is an important disinfectant in hospitals and food industries. Iodophors such as povidone-iodine solution are commonly used as chemically synthesized iodine disinfectants (6). Recently, however, research efforts to evaluate natural antimicrobial compounds, including enzymes with antimicrobial activity, have increased (15). Hickey et al. (21) reported that horseradish peroxidase could be used to generate enough I_2 to kill *Staphylococcus aureus* and the spores of *B. subtilis*. Hansen et al. (18) also found that haloperoxidase isolated from *Curvularia verruculosa* had the potential to kill a variety of bacteria, yeasts, and fungi in the presence of bromide or iodide. These haloperoxidase-based systems, however, have the disadvantage of requiring hazardous H_2O_2. As discussed above, IOE-II is an oxidase and does not need H_2O_2 for I_2 production. Therefore, it is possible that IOE-II/iodide can be used as a simple, safe, and cost-effective antimicrobial enzyme system. Determination of its antimicrobial spectrum and comparison of its antimicrobial capacity with other disinfectants are under way in our laboratory.

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